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## Antidepressant phenotype by inhibiting the phospholipase C $\beta_1$ – Protein kinase C $\gamma$ pathway in the forced swim test

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### ABSTRACT

Although great advances have recently been made in the study of signal transduction, the pathogenesis of affective disorders is still unknown. There is mounting evidence suggesting that elevated phosphoinositide–protein kinase C (PI–PKC) signal transduction pathway may be a pathophysiological feature of bipolar and major depressive disorders. The aim of the present study was to further investigate the phospholipase C–protein kinase C (PLC–PKC) cascade by evaluating the effect produced by an acute blockade of this intracellular pathway at PLC and PKC level. Adult male mice were administered with pharmacological inhibitors of PLC or PKC and then subjected to the forced swim test (FST), an animal model which emulates the behavioural despair paradigm of depression. In this study we also tested the hypothesis that it might be possible to selectively modulate depressive behaviour by inhibiting the expression of specific PLC and PKC isoforms by means of specific antisense oligonucleotides (aODNs). Administration of the PLC inhibitors neomycin and U73122 as well as of the PKC inhibitors calphostin C and chelerytrine dose-dependently reduced the immobility time in the FST producing an antidepressant-like behaviour. Selective knockdown of the PLC $\beta_1$  and PKC $\gamma$  isoforms also induced an antidepressant phenotype. Conversely, the inhibition of the expression of PLC $\beta_3$  was unable to modify the immobility time values. The PLC and PKC modulators used, at the highest effective doses, altered neither locomotor activity nor motor coordination. We demonstrate that selective blockade of PLC $\beta_1$ –PKC $\gamma$  signalling pathway produces an antidepressant-like phenotype in mice.

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### 1. Introduction

Research into the pathophysiology of affective disorders and the mechanism of action of antidepressant drugs has primarily focused on altered monoamine neurotransmission. However, extensive studies have failed to prove conclusively abnormalities in neurotransmitter or post-synaptic receptor function. This has led to an examination of post-receptor signal transduction systems (Marazziti et al., 2009).

There is mounting evidence suggesting that an alteration of the phosphoinositide–protein kinase C (PI–PKC) signal transduction pathway may be associated to affective disorders. In the PI–signalling system, agonists' stimulation of G protein coupled receptors causes hydrolysis of the substrate, phosphatidyl inositol

4,5-bisphosphate (PIP<sub>2</sub>), by the enzyme phospholipase C (PLC), resulting in the formation of two second messengers, inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) (Berridge and Irvine, 1989). IP<sub>3</sub> stimulates the release of intracellular calcium from the endoplasmic reticulum, and DAG stimulates the enzyme protein kinase C (PKC) (Nishizuka, 1992).

Platelets from patients with bipolar disorders (Brown et al., 1993; Soares et al., 2001) or major depressive disorders (Dwivedi et al., 1998; Karege et al., 1996) exhibit several indices consistent with an increased PI activity, implying that depression is associated with over-stimulation of the PI pathway in platelets. More direct evidence of an increased PI-related signal transduction in affective disorders comes from studies in post-mortem human brain tissues (Friedman and Wang, 1996). In particular, these alterations might be a consequence of increased PI levels (Silverstone et al., 2005) or G $\alpha_{q/11}$  and PLC immunoreactivity (Mathews et al., 1997). An elevated PKC signalling activity has also been reported in platelets (Wang et al., 1999; Pandey et al., 1998) and brain samples (Wang and Friedman, 1996) from depressed patients. A further confirmation of this hypothesis is given by the observation that antidepressant drugs from different classes down-regulate the PI–PKC

Abbreviations: aODN, Antisense oligonucleotide; FST, Forced swim test; i.c.v., Intracerebroventricular; PI–PKC, Phosphoinositide–protein kinase C; PLC, Phospholipase C; PKC, Protein kinase C.

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signal transduction pathway by reducing PLC expression (Dwivedi et al., 2002), desensitising  $G\alpha_q$ -coupled receptor-generated PLC-mediated hydrolysis of  $PIP_2$  (Dyck and Boulton, 1989; Pandey et al., 1991) or decreasing PKC activity (Mann et al., 1995; Morishita and Watanabe, 1997) in animal and human studies.

On this matter, there is not a general consensus. In contrast to the hypothesis of an over-stimulated PI cascade, it has been suggested that a hypofunctionality, rather than a hyperactivity, of the PI–PKC pathway might be related to affective disorders. Studies performed in platelets and human brain samples reported no difference (Coull et al., 2000) or decreases in indices of PI or PKC signalling (Jope et al., 1996; Pandey et al., 2002) in patients suffering from bipolar or major depressive disorders. Specifically, a decreased activity and expression of PLC and PKC have been observed in teenage suicide victims (Pandey et al., 1999, 2004).

As a step towards better understanding of the involvement of PI–PKC signalling pathway in mood disorders, we further investigated the PLC–PKC cascade by evaluating the effect produced by a pharmacological blockade of this intracellular pathway in mice exposed to the forced swim test, an animal model which emulates the behavioural despair paradigm of depression. In this study we also tested the hypothesis that it might be possible to selectively modulate depressive behaviour in antidepressant-responsive behavioural paradigms by inhibiting the expression of specific PLC and PKC isoforms by using an antisense strategy.

## 2. Materials and methods

### 2.1. Animals

Male Swiss albino mice (20–22 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Ten mice were housed per cage (26 × 41 cm). The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at  $23 \pm 1^\circ\text{C}$  with a 12 h light/dark cycle, light on at 7 a.m. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimise animal suffering, and to reduce the number of animals used.

### 2.2. Intracerebroventricular injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anaesthesia, as previously described (Galeotti et al., 2003). Briefly, during anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10  $\mu\text{l}$  syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5  $\mu\text{l}$  were then administered. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5  $\mu\text{l}$  of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was determined to be 95%. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5  $\mu\text{l}$  per mouse.

### 2.3. Forced swim test

The forced swimming test used was the same as described by Porsolt et al. (1977). Briefly, mice were placed individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 6 cm of water maintained at  $22\text{--}23^\circ\text{C}$  and left there for 6 min. A mouse was judged to be immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect. 12–15 mice per group were tested.

### 2.4. Motor coordination

The motor coordination was assessed by using the rota rod test. The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were

tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. Those mice scoring less than 3 and more than 6 falls in the pretest were rejected (20%). The number of falls was measured before (pretest) and 15, 30 and 45 min after the beginning of the test. 10 mice per group were used.

### 2.5. Locomotor activity

The locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. 12–15 mice per group were tested.

### 2.6. Antisense oligonucleotides

Phosphodiester oligonucleotides (ODNs) protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were obtained from Tib Molbiol (Genoa, Italy). The following aODN was produced against  $PLC\beta_3$ : 5'-T\*G G\*TTGGTCATCTGGGATG\*T\*A-3' where\* indicates the phosphorothioate residue. Anti- $PLC\beta_3$  ODN was designed from the sequence 2649–2669 of the gi: 31982121 NCBI Sequence Viewer; this sequence was searched through GenBank and found to be unique to its respective  $PLC\beta_3$  isoenzyme and was based on mouse sequence. The aODN against  $PKC\gamma$  was the following: 5'-A\*C\*GAAGTCCGGGTTTACA \*T\*A-3'. Anti- $PKC\gamma$  ODN was designed from the sequence 2359–2378 of the gi: 31982442 NCBI Sequence Viewer; this sequence was searched through GenBank and found to be unique to its respective  $PKC\gamma$  isoenzyme and was based on mouse sequence. The aODN against  $PLC\beta_1$  was the following: 5'-G\*C\*T GTC GGA CAC G\*C\*A -3', corresponding to nucleotides 49–63 of the  $PLC\beta_1$  gene sequence. Anti- $PLC\beta_3$  was previously characterised by *in vitro* and *in vivo* experiments in our laboratory (Galeotti et al., 2006). Anti- $PLC\beta_1$  was previously characterised by Sanchez-Blazquez and Garzon (1998) and by us. We confirmed the aODN effect on  $PLC\beta_1$  protein levels by performing immunoblotting experiments. We observed a statistically significant reduction of the expression of  $PLC\beta_1$  ( $45.1 \pm 6.9$ ) after aODN treatment in comparison with mice treated with dODN. The 15 and 20mer fully degenerated ODNs (dODNs), where each base was randomly G, or C, or A, or T, were used as control ODN treatment. Antisense ODNs and dODNs were pre-incubated at  $37^\circ\text{C}$  for 30 min with an artificial cationic lipid (13  $\mu\text{M}$  DOTAP, Sigma, Milan, Italy) and i.c.v. injected to mice in a 5  $\mu\text{l}$  final volume. Mice received a single i.c.v. injection on day 1, 2 and 3. Behavioural and western blotting experiments were performed on day 4, 24 h after the last i.c.v. injection.

### 2.7. Preparation of membranes

Brain areas to conduct western blotting experiments were collected 24 h after the end of the anti- $PKC\gamma$  i.c.v. treatment. Mouse brains were dissected to separate specific areas. Mouse hippocampus and frontal cortex were homogenised in a homogenisation buffer containing 25 mM Tris–HCl pH = 7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPPi, 4 mM PNFF, 1 mM  $\text{Na}_2\text{VO}_4$ , 1 mM PMSF, 20  $\mu\text{g}/\text{ml}$  leupeptin, 50  $\mu\text{g}/\text{ml}$  aprotinin, 0.1% SDS. The homogenate was centrifuged at  $9000 \times g$  for 15 min at  $4^\circ\text{C}$ , the low speed pellet was discarded and the supernatant was stored at  $-80^\circ\text{C}$ . Protein concentration was quantified using Bradford's method (protein assay kit, Bio-Rad Laboratories, Milan, Italy).

### 2.8. Western blot analysis

Membrane homogenates (10  $\mu\text{g}$ ) made from hippocampus and frontal cortex regions of dODN and aODN treated mice were separated on 10% SDS–PAGE and transferred onto nitrocellulose membranes (110 min at 120 V) using standard procedures. Membrane were blocked in PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 120 min. Following washings, blots were incubated overnight at  $4^\circ\text{C}$  with specific antibodies against  $PKC\gamma$  (1:1000; Santa Cruz Biotechnology Inc, CA, USA) or  $\beta$ -actin (1:1000 dilution). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10,000) and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy). Exposition and developing time used was standardised for all the blots. Densitometric analysis of scanned images was performed on a Macintosh iMac computer using the public domain NIH Image program. Measurements in control samples were assigned a relative value of 100%. Measurements were normalised relative to  $\beta$ -actin, used as loading control.

### 2.9. Drug administration

The following drugs were used: calphostin C, chelerytrine, neomycin, U73122 (1-[6-((17 $\beta$ -3-methoxyestra-4,3,5(10)-trien-17-yl)amino)hexyl]1H-pyrrole-2,5-dione), U73343 (1-[6-[[17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrroli-dinedione), PMA (phorbol-12-myristate-13-acetate), PDBu (phorbol-12,13-dibutyrate) (Calbiochem, Milan Italy); *D*-amphetamine hydrochloride (De Angeli, Rome, Italy); amitriptyline (Sigma, Milan, Italy). *D*-amphetamine, amitriptyline and neomycin were dissolved in isotonic (NaCl 0.9%) saline solution, U73122, U73343, calphostin C, chelerytrine, PMA, PDBu in 0.5% DMSO. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5  $\mu$ l per mouse by intracerebroventricular (i.c.v.) injection and 10 ml kg<sup>-1</sup> by subcutaneous (s.c.) or intraperitoneal (i.p.) injection. PMA, PDBu, chelerytrine and Calphostin C were injected i.c.v. 1 h before the test; U73122, neomycin and *D*-amphetamine (2 mg kg<sup>-1</sup> s.c.) were injected 10 min before behavioural tests, amitriptyline (10 mg kg<sup>-1</sup> i.p.) was injected 30 min before the tests. Doses and administration schedules were chosen on the basis of time-course and dose-response curves performed in our laboratory (Galeotti et al., 2003). Furthermore, literature data confirm the selectivity and efficacy of the above-mentioned treatments at time and concentration used.

### 2.10. Statistical analysis

All experimental results are given as the mean  $\pm$  s.e.mean. Analysis of variance (one-way ANOVA) followed by Bonferroni/Dunn post hoc test was used for statistical analysis.

## 3. Results

### 3.1. Antidepressant-like effect induced by inhibition of phospholipase C (PLC)

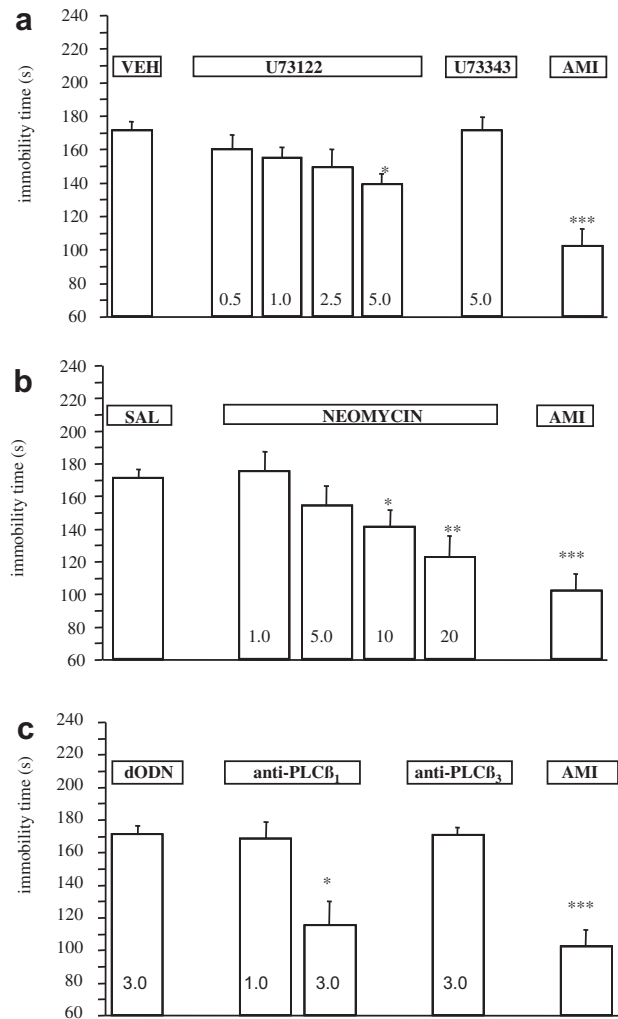
The effect produced by blockade of the PLC in a behavioural despair paradigm was determined in the mouse forced swimming test (FST). A reduction of the immobility was produced by the PLC blocker U73122 (F(7,93) 4.750  $p$  < 0.001). A statistically significant effect was reached at the doses of 5  $\mu$ g ( $p$  < 0.05) per mouse i.c.v. (Fig. 1a). Treatment with U73343, analogue of U73122 ineffective on PLC, at the dose of 5  $\mu$ g per mouse i.c.v. was devoid of any effect (Fig. 1a). The i.c.v. administration of the PLC inhibitor neomycin dose-dependently reduced the immobility time (F(5,78) 5.940  $p$  < 0.0001). Bonferroni/Dunn multiple comparison analysis showed that the administration of 10 ( $p$  < 0.05) or 20  $\mu$ g ( $p$  < 0.01) per mouse i.c.v. produced a statistically significant antidepressant-like effect (Fig. 1b).

The reduction of the expression of the PLC $\beta_1$  isoform by aODN pretreatment induced an antidepressant-like phenotype (F(3,48) 3.937  $p$  < 0.001). The dose of anti-PLC $\beta_1$  of 1 nmol per mouse i.c.v. was ineffective whereas the dose of 3 nmol per mouse reached the statistical significance ( $p$  < 0.05; Fig. 1c). Conversely, the knock-down of the PLC $\beta_3$  isoform did not modify the immobility time values in comparison with the dODN-treated group (F(1,23) 1.717  $p$  = 0.227) (Fig. 1c).

In the same experimental conditions, amitriptyline (10 mg kg<sup>-1</sup> s.c.), used as reference drug, produced an antidepressant-like effect.

### 3.2. Antidepressant-like effect induced by inhibition of protein kinase C (PKC)

The i.c.v. administration of calphostin C, a PKC blocker, decreased the immobility time values in the mouse FST. Calphostin C, at 0.001  $\mu$ g per mouse i.c.v., was devoid of any effect, whereas the doses of 0.01 and 0.02  $\mu$ g per mouse decreased the immobility time without reaching the statistical significance. The ANOVA on the immobility time revealed a significant group effect (F(6,77) 11.332;  $p$  < 0.0001). Bonferroni/Dunn multiple comparison analysis showed that the administration of either 0.05 ( $p$  < 0.05) or 0.1  $\mu$ g ( $p$  < 0.01) per mouse i.c.v. induced a statistically significant



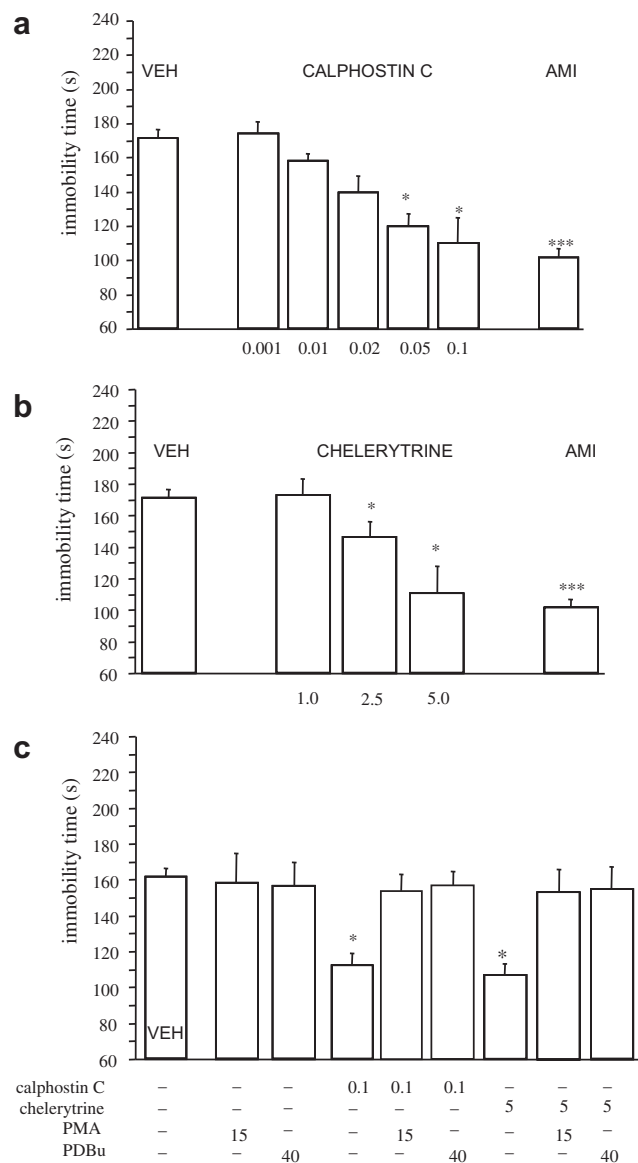
**Fig. 1.** Antidepressant-like effect produced by blockade of the PLC activity. Dose-dependent decrease of the immobility time in the mouse forced swimming test by the pharmacological PLC blockers U73122 (0.5–5  $\mu$ g per mouse i.c.v.) (a) and neomycin (1–20  $\mu$ g per mouse i.c.v.) (b). Effect produced by knockdown of PLC $\beta_1$  and PLC $\beta_3$  (c). Amitriptyline (AMI, 10 mg kg<sup>-1</sup> i.p.) was used as reference drug. VEH: 0.5% DMSO; SAL: saline; dODN: degenerate ODN. Vertical lines represent SEM. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 in comparison with control mice.

antidepressant-like effect (Fig. 2a). Similarly, the i.c.v. administration of the PKC blocker chelerytrine produced an antidepressant-like effect (F(4,63) 56.498;  $p$  < 0.001). The dose of 1  $\mu$ g per mouse i.c.v. was ineffective whereas the statistical significance (Bonferroni/Dunn multiple comparison analysis) was reached at 2.5 ( $p$  < 0.01) and 5 ( $p$  < 0.0001)  $\mu$ g per mouse i.c.v. (Fig. 2b).

The intensity of the calphostin C, and chelerytrine antidepressant-like effect was comparable to that produced by amitriptyline (10 mg kg<sup>-1</sup> s.c.), used as reference drug.

Co-administration of calphostin C (0.1  $\mu$ g per mouse i.c.v.) with the PKC activators PMA (15 nmol per mouse i.c.v.) or PDBu (40 nmol per mouse i.c.v.) prevented the calphostin C-induced reduction of the immobility time up to control values (Fig. 2c). Similarly, PMA and PDBu prevented the antidepressant-like effect of chelerytrine (5  $\mu$ g per mouse i.c.v.) (Fig. 2c). PMA and PDBu, when administered alone, were devoid of any effect on immobility time (Fig. 2c).

PKC $\gamma$  isoform protein knockdown induced an antidepressant-like phenotype (F(3,52) 4.217  $p$  < 0.01), even if of lower intensity than that produced by pharmacological blockers of PKC. The dose of

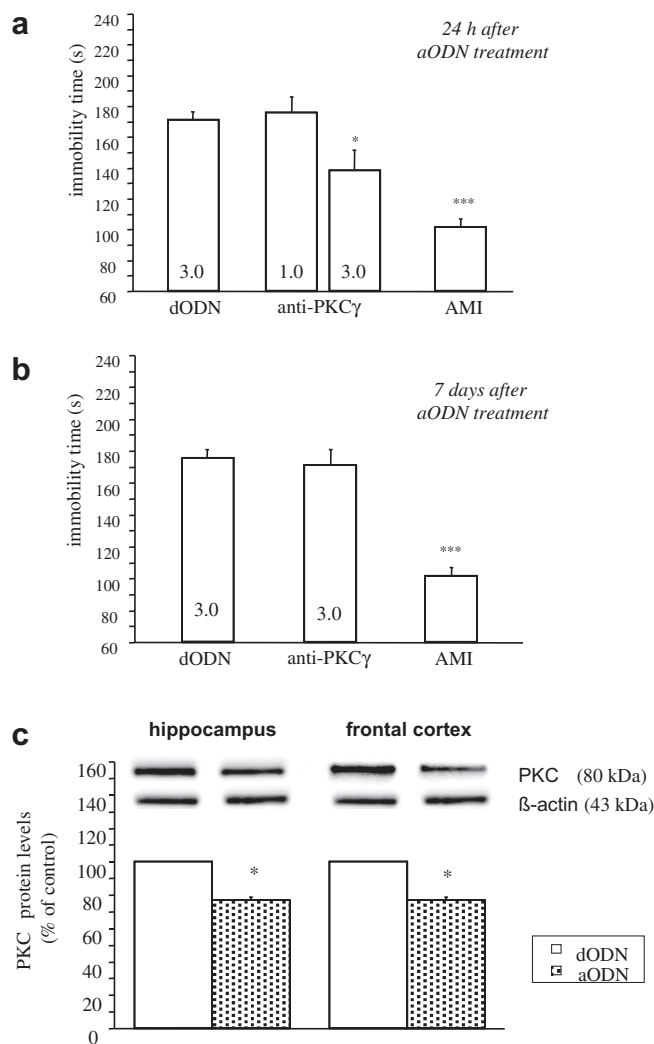


**Fig. 2.** Antidepressant-like effect produced by pharmacological blockade of PKC activity. Dose-dependent decrease of the immobility time in the mouse forced swimming test by the PKC blockers calphostin C (0.001–0.1 μg per mouse i.c.v.) (a) and chelerytrine (1–2.5 μg per mouse i.c.v.) (b) in comparison with amitriptyline (AMI, 10 mg kg<sup>−1</sup> i.p.), used as reference drug. (c) Prevention by pretreatment with the PKC activators PMA (15 pmol per mouse i.c.v.) and PDBu (40 pmol per mouse i.c.v.) of the antidepressant-like effect induced by calphostin C (0.1 μg per mouse i.c.v.) and chelerytrine (5 μg per mouse i.c.v.). VEH: 0.5% DMSO. Vertical lines represent SEM. \**p* < 0.05, \*\*\**p* < 0.001 in comparison with control mice.

anti-PKCγ of 1 nmol per mouse was ineffective; the statistical significance was reached pre-treating animals with the dose of 3 nmol per mouse i.c.v. (Fig. 3a). Animals exposed to an FST session 7 days after the end of the aODN treatment showed the absence of any antidepressant-like activity as indicated by the immobility time values that were comparable to those produced by the control group (Fig. 3b).

### 3.3. Effect of aODN on PKCγ protein levels

Mice were treated with the aODN on day 1, 2 and 3. On day 4, 24 h after the last i.c.v. injection, mice were killed and the



**Fig. 3.** Antidepressant-like effect produced by knockdown of PKCγ following administration of a specific antisense oligonucleotide (anti-PKCγ). (a) Decrease of the immobility time in the mouse forced swimming test by anti-PKCγ (1–3 nmol per mouse i.c.v.) 24 h after the end of the aODN treatment. (b) Reversibility of the antidepressant-like phenotype 7 days after the end of the aODN treatment (3 nmol per mouse i.c.v.). Amitriptyline (AMI, 10 mg kg<sup>−1</sup> i.p.) was used as reference drug; dODN: degenerate oligonucleotide. Vertical lines represent SEM. \**p* < 0.05, \*\*\**p* < 0.001 in comparison with control mice. (c) Reduction of PKCγ protein expression in mouse cortex and hippocampus by aODN treatment in comparison with dODN-treated mice. Samples (10 μg protein/lane) of mouse cortex and hippocampus were resolved on 10% SDS–PAGE, transferred to nitrocellulose and probed with PKCγ antibody. β-actin was used as loading control. The columns represent the densitometric quantitation of immuno-reactive protein expressed relative to control. Data are expressed as mean ± SEM of band intensities from each group (*n* = 6 per group). Representative immuno-blot were reported above each column. \**p* < 0.05 versus control.

hippocampus and frontal cortex were dissected and examined for the levels of expression of PKCγ in comparison with mice treated with dODN. Fig. 3c shows a representative immunoblot where a prominent approximately 80 kDa protein band was observed, indicating that PKCγ was expressed in the above-mentioned cerebral areas. The dODN did not significantly change the level of immunostaining when compared with that of naive animals (data not shown). Densitometric analysis of all samples revealed a statistically significant reduction of the expression of PKCγ after aODN treatment in comparison with mice treated with dODN in both cerebral areas (Fig. 3c). Levels of PKCγ protein expression returned to normal 7 days after the end of the aODN treatment (data not shown).



### 3.4. Effect of PLC and PKC modulators on mouse motor coordination and locomotor behaviour

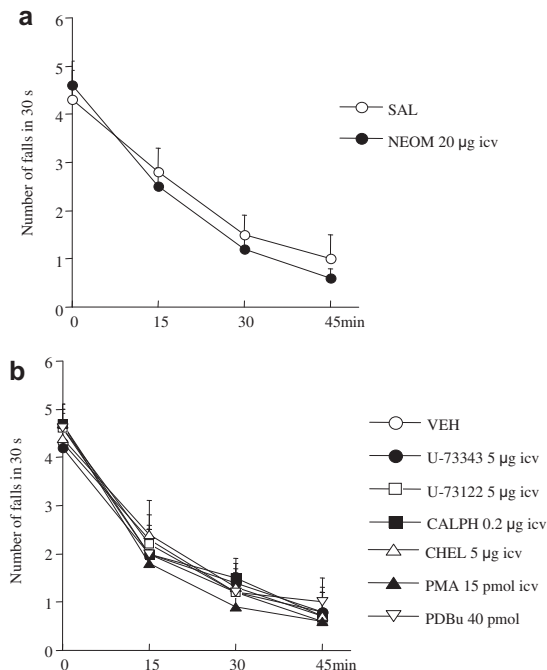
Mice pretreated with the PLC and PKC inhibitors were evaluated for motor coordination and locomotor behaviour by use of the rota rod and hole-board tests, respectively.

The number of falls from the rotating rod, evaluated before and 15, 30 and 45 min after the beginning of the rota rod test, showed the lack of any alteration in the motor coordination following administration of the highest effective doses of the PLC inhibitors neomycin (20 µg per mouse i.c.v.) and U73122 (5 µg per mouse i.c.v.), the PKC inhibitors calphostin C (0.2 µg per mouse i.c.v.), chelerytrine (5 µg per mouse i.c.v.) and the PKC activators PMA (15 nmol per mouse i.c.v.) and PDBu (40 nmol per mouse i.c.v.), in comparison with the corresponding control group (Fig. 4a,b).

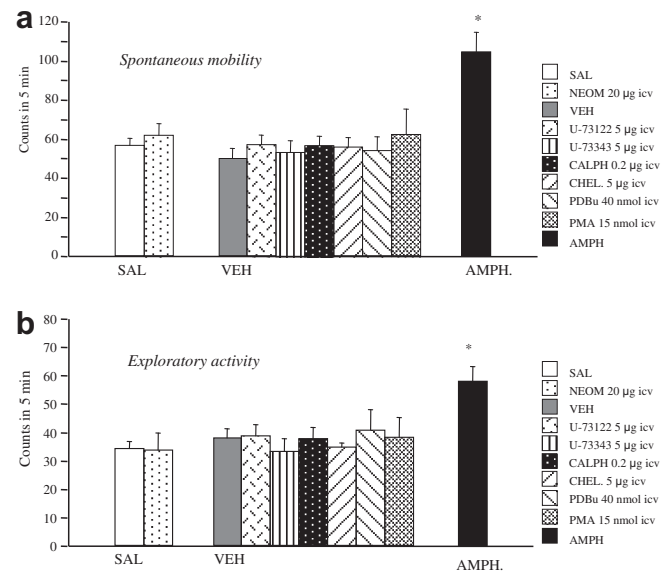
The spontaneous mobility (Fig. 5a) and exploratory activity (Fig. 5b) of mice treated with the highest effective doses of the above-mentioned compounds were unmodified in comparison with the control group. In the same experimental conditions D-amphetamine, used as positive control, significantly increased both spontaneous mobility and exploratory activity (Fig. 5a,b).

## 4. Discussion

The role of PI–PKC signalling pathway in the molecular mechanism of depression has increasing consensus, but data are often controversial and still await further investigation. We, therefore, studied the involvement of the PLC–PKC pathway in a behavioural despair paradigm, the mouse forced swimming test (FST), providing evidence for a role of PLC and PKC in the induction of an antidepressant-like condition. FST is a putative animal model of depression, which emulates the behavioural despair paradigm of depression. Currently it is one of the most frequently used



**Fig. 4.** Lack of alteration of mouse motor coordination by PLC and PKC modulators. The absence of any modification in the number of falls from the rotating rod by the PLC inhibitors neomycin (NEOM) (a) and U73122, the PKC inhibitors calphostin C (CALPH) and chelerytrine (CHEL), and the PKC activators PMA and PDBu (b) was observed in comparison with corresponding control group. SAL: saline; VEH: 0.5% DMSO. Vertical lines represent SEM.



**Fig. 5.** Lack of influence on mouse locomotor activity by PLC and PKC modulators. The absence of any alteration of (a) spontaneous mobility (b) and exploratory activity by the PLC inhibitors neomycin (NEOM) and U73122, the PKC inhibitors calphostin C (CALPH) and chelerytrine (CHEL), and the PKC activators PMA and PDBu was observed in comparison with corresponding control group. Amphetamine (amph) was used as positive control. SAL: saline; VEH: 0.5% DMSO. Vertical lines represent SEM. \* $p < 0.05$  in comparison with control group.

behavioural tests for investigating antidepressant potential that is sensitive to acute drug administration, and provides a high degree of pharmacological predictive validity (Petit-Demouliere et al., 2005).

Several PLC inhibitors are currently available. U73122, an aminosteroid, has been found to be a potent inhibitor of aggregation of human platelets induced by a variety of agonists, and this compound has been further characterised as an inhibitor of G protein-mediated PLC (Wakdo et al., 1983; Yule and Williams, 1992). Neomycin has been reported to inhibit hormone-stimulated IP<sub>3</sub> production through the blockade of PLC (Phillippe, 1994). A single i.c.v. administration of the specific PLC inhibitors U73122 and neomycin produced an antidepressant-like response of intensity comparable to that induced by amitriptyline, taken as antidepressant reference drug. An unspecific effect of the treatments can be excluded since, U73343, a succinimido analogue used as negative control for U73122 being a weak inhibitor of PLC (Bleasdale et al., 1990; Smith et al., 1990), did not modify the immobility time values in comparison with the control group. These results indicate that inhibition of the PLC activity produces an antidepressant-like behaviour further suggesting that the stimulation of the PLC-mediated pathway might be involved in affective disorders.

On the basis of sequence homology and localisation of structural domains PLC has been characterized into three major families:  $\beta$ ,  $\delta$  and  $\gamma$  (Rebecchi and Pentylala, 2000; Rhee, 2001). All PLC isozymes recognise PIP<sub>2</sub> as a substrate and carry out Ca<sup>2+</sup>-dependent hydrolysis of inositol lipids; however these enzymes are differentially regulated and expressed. PLC $\beta$  is activated by receptors that activate the Gq family of the G proteins, whereas PLC $\gamma$  is regulated by receptors and non-receptor tyrosine kinases (Rhee, 2001). Little is known about the regulation of PLC $\delta$ . The PLC $\beta$  family has been divided into 4 isoforms:  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  and  $\beta_4$ . The PLC $\beta_1$ ,  $\beta_3$  and  $\beta_4$  are expressed in the brain whereas the PLC $\beta_2$  has a poor cerebral expression (Ross et al., 1989; Tanaka and Kondo, 1994). Among the PLC $\beta$  isoforms, the antisense-induced knockdown of the PLC $\beta_1$  produced an antidepressant-like phenotype in contrast to

the inhibition of the expression of the PLC $\beta_3$  isoform, which was devoid of any effect on the immobility time. These results indicate a prominent role of the PLC $\beta_1$  isoform in the modulation of depressive conditions and identify the inactivation of this PLC isoform as a potential strategy for antidepressant therapy. This hypothesis is supported by literature data indicating that antidepressant drugs from different classes specifically decreased PLC $\beta_1$  protein levels in rat cortex and hippocampus (Dwivedi et al., 2002).

PLC isozymes hydrolyse the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate generating two intracellular products: IP $_3$ , a universal calcium-mobilising second messenger, and diacylglycerol (DAG), an activator of PKC. PKC is a family of phosphorylating enzymes that are divided into three groups based on calcium and DAG dependence: conventional ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), atypical ( $\zeta$ ,  $\lambda$ /I) (Way et al., 2000). The translocation of these enzymes from the cytosol to the synaptic membrane is thought necessary for their activation (Nishizuka, 1992). In the present study, pretreatment with calphostin C and chelerytrine, selective, potent and membrane-permeable PKC inhibitors (Kobayashi et al., 1989; Herbert et al., 1990) produced an antidepressant-like behaviour showed by a dose-dependent reduction of the immobility time values in the FST. Furthermore, activation of PKC by phorbol esters, such as PMA and PDBu (Nishizuka, 1992), prevented the antidepressant activity produced by the PKC blockers confirming that the antidepressant phenotype produced by calphostin C and chelerytrine underlie a selective blockade of PKC. Recently, it has been demonstrated that the PKC inhibitor tamoxifen reduces the amphetamine-induced hyperlocomotion, an animal model of mania (Einat et al., 2007; Sabioni et al., 2008), and produces an anti-manic effect in patients with bipolar disorders (Zarate et al., 2007; Yildiz et al., 2008), supporting the possibility that PKC signalling pathway may play an important role in the pathophysiology and treatment of bipolar disorders. Present results clearly indicate that blockade of the PKC-mediated pathway is related to the induction of an antidepressant phenotype, suggesting that PKC is not only confined to the induction/treatment of mania in bipolar disorders. This hypothesis is also supported by the observation that repeated administration of fluoxetine and desipramine, antidepressant drugs effective in the treatment of major depression and dysthymia, decreased the basal activity of soluble and particulate PKC in rat cerebral cortex and hippocampus (Mann et al., 1995) and in platelets from affective disorders subjects (Morishita et al., 1999). The activation of PKC might, therefore, constitute a significant pathway involved in the pathophysiology of affective disorders and its inactivation might represent an important therapeutic approach.

PKC is highly expressed in the brain and the expression is isoform- and region-specific, suggesting that the enzyme is involved in selective cellular functions. Among the PKC isoform, PKC $\gamma$  is solely expressed in the central nervous system, where it is particularly concentrated in the hippocampus and cortex (Saito et al., 1988), brain regions most likely to be implicated in response to depression. There are morphological and functional alterations in the both brain regions in humans with major depression, such as reduced volume of the hippocampus (Bremner, 2002), and altered metabolism in the prefrontal cortex (Drevets et al., 1997). We, then, decided to investigate the involvement of the PKC $\gamma$  isoform in mood disorders. The inhibition of the expression of PKC $\gamma$  following administration of a specific antisense oligonucleotide (aODN) reduced the immobility time values, indicating the presence of an antidepressant-like phenotype. On the bases of these observations, it is plausible to suppose that an over-stimulation of the PKC $\gamma$  might be present in depressed patients and that the inactivation of PKC $\gamma$  may represent a new target for the antidepressant therapy. However, a decreased catalytic activity and expression of PKC $\gamma$  in

prefrontal cortex and hippocampus of teenage suicide victims has been observed (Pandey et al., 2004). This discrepancy might be explained by taking into consideration that the neurobiology of teenagers is different from that of adults, since the observation that responses to antidepressant treatments (Hazell et al., 1995; Emslie et al., 1997) and risk factors for suicide (Apter et al., 1995; Brent et al., 1993) differ between adolescents and adults with depression.

The capability of the aODN treatment to knockdown PKC $\gamma$  was demonstrated by immuno-blotting experiments where a selective decrease of PKC $\gamma$  protein levels was detected in the hippocampus and frontal cortex. These results showed that the antidepressant phenotypic effect was related to a selective knockdown of the PKC $\gamma$  isoform. The degenerate ODN (dODN), used as control for the unspecific effects attributable to the administration of nucleic acids irrespective of their specific sequence, did not modify the immobility time in comparison with naive or vector-injected mice. This observation ruled out the possibility that the antidepressant behaviour showed by PKC $\gamma$  knockdown animals may have resulted from a sequence-independent action on cerebral structures. The antidepressant-like effect induced by anti-PKC $\gamma$  disappeared 7 days after the end of the antisense administration, indicating an absence of irreversible damage or toxicity on cerebral structures caused by the aODNs.

The FST is widely used to predict the antidepressant action of drugs in humans. However, this animal model has also some drawbacks represented by the possibility to obtain some false positives or negatives by drugs that modify motor activity, which, therefore, may alter the immobility time values recorded. The reduced immobility time observed following injection of PLC and PKC modulators was not accompanied by any modification of spontaneous mobility and locomotor activity of mice, potential confounds for the evaluation of a depressant/antidepressant effect.

Present results demonstrate that acute pharmacological blockade of PLC $\beta_1$ –PKC $\gamma$  signalling pathway produces an antidepressant-like phenotype in the mouse FST. These findings suggest the PLC–PKC cascade as critical target underlying depressive states and should facilitate the comprehension of the etiopathology of mood disorders and help developing new therapeutic strategies.

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